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Sporulation and the Production of Antibiotics, Exoenzymes, and Exotoxins

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INTRODUCTION

This article has two purposes: to illustrate the usefulness of asporogenous mutants in studying the mechanism of the sporulation process, and to discuss the relationship between this process and the production of antibiotics, exoenzymes, and exotoxins. It consists of three parts. In the first, of an introductory nature, relevant information on sporulation in bacteria is mentioned briefly. A number of important points are left out, which are very adequately covered in recent reviews (76, 77, 133, 201). The main part of this review is the second one, which reports and discusses the

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work on asporogenous bacterial mutants up to the summer of 1968. A mere addendum to the second part, the third is an attempt to evaluate, from a survey of the literature, the possible extension to actinomycetes and lower fungi of the conclusions reached from work on bacteria. Successive approaches to this presentation have already been published (172–175, 179).

SPORULATION IN EUBACTERIA

Sporulation of bacilli and clostridia is considered here in its cytological, physiological, and biochemical aspects; the genetic approach is also discussed.

Cytological Aspect

Successive changes in cell structure which occur during sporulation were first described in *Bacillus cereus* by Young and Fitz-James (240, 241); their description is generally valid for other species (18, 82, 159, 165). These changes are continuous, but it is convenient to select a few typical stages (Fig. 1A) and ascribe a number to each (61, 179). Cells are said to be at stage I when the chromatin is visible as a single axial rod, rather than the one or two nuclear bodies observed in the growing cell (168). Stage II begins with an invagination of the cytoplasmic membrane and ends with the completion of a spore septum, dividing the nuclear material in two, and creating a double-celled organism, the sporangium. This septum is easily distinguished from a division septum by its location close to a pole of the cell and by the absence of wall material between the two membrane layers. Further elongation of the double membrane, which bulges into the bigger part of the cell, is accompanied by a polewards displacement of the insertion point of the septum, until an entirely intracytoplasmic forespore is "carved out" of the mother cell (state III). The next two stages are characterized by the formation of new envelopes around the forespore: the cortex, formed between the two membranes at stage IV, and the two layers of the spore coat, exterior to the cortex, formed at stage V. A change in the structure of the cortex then occurs, as the thermoresistance of the enclosed spore is being built up (stage VI). Lysis of the mother cell eventually sets the mature spore free. Schematic representations of the various stages, as observed in *B. subtilis*, are presented in Fig. 1A.

Physiological Aspect: Catabolic Repression of Sporulation

Unless mentioned otherwise, the following observations apply to the Marburg strain of *B. subtilis*. Nutrient broth cultures, continuously aerated at 37 C, yield a full crop of spores (at least 60% of the population present at t_0) 6.5 to 7 hr after the end of exponential growth, arbitrarily taken as the t_0 of sporulation (179). The times at which cells at a given stage of their development predominate in the culture are also indicated in Figure 1A. If enough glucose to support growth for 2 hr is added to the culture at t_0, growth continues at an undisturbed rate; thus, nitrogen is in excess in the medium. Under such conditions, less than 1% of the cells are found as thermoresistant spores at $t_{6.5}$. Addition of glucose at $t_{0.5}$, however, does not reduce the yield of spores at $t_{6.5}$; by that time, apparently, all the cells that were to sporulate were committed to do so (64, 69, 70). Two remarks are in order here, the first on Foster's definition of commitment. A cell is considered to be committed to sporulate at time t, if glucose addition at that time no longer prevents it from forming a mature spore in due time (64). If the medium contains a nitrogen source, vegetative cells increase in number after addition of glucose. This is because a few per cent of the cells fail to sporulate, escape commitment, and continue to grow in the enriched medium. Also, up to stage II inclusively, the sporangium of a committed cell is still able to resume its multiplication (61, 239). If enough glucose is added, however, these newly formed cells will not contribute to the yield of spores at t_7. Incidently, resumption of growth from the nonsporal part of a sporangium shows that its nucleus, too, contains at least one complete genome (239).

The other remark concerns the notion of t_0. It is nothing more than an admittedly arbitrary but convenient time mark, which cannot be devoid of physiological meaning, since it is the time of exhaustion of the growth substrate. Its use does not necessarily imply, however, that no sporulation-specific event took place previously, even in an ideally synchronized culture. The time of commitment might conceivably be taken also as an arbitrary t_0, but cannot be determined in the course of an experiment. Whereas the adoption of a t_0 of sporulation has been found useful, Wright's warning (230) that a differentiation process may have no t_0 at all is to be kept in mind.

In contrast to *B. subtilis*, starvation of *B. megaterium* does not promote cell lysis. As is well known, sporulation occurs when either carbon or nitrogen source, or both, are lacking (69–71). Transfer experiments (64) were made with *B. subtilis* cells grown in broth, filtered, and resuspended in mineral medium lacking either a carbon or a nitrogen source. Lysis by starvation (128) occurred in the C^-N^+ medium, but good sporulation was often obtained in the C^+N^- medium (*unpublished data*). Thus, it appears that, at least in

B. megaterium and in *B. subtilis*, sporulation is repressed by glucose only in the presence of a utilizable nitrogen source (i.e., under conditions supporting growth). Perhaps some *Bacillus* species draw more easily on their intracellular nitrogen sources than others, since glucose alone in some cases seems sufficient to repress sporulation (64).

In *B. megaterium*, spores are formed during exponential growth on glucose-mineral medium; their number increases in parallel with that of the viable cells (9), the spore-to-cell ratio at equilibrium depending on the nature of the carbon source (6). Experiments with *B. subtilis* confirmed this finding and showed this ratio to be dependent also on the nature of the nitrogen source (180). Many amino acids, added singly to the minimal medium, lower the ratio considerably (down to 0.1% of the minimal-medium value in the case of glutamic acid); their sporulation-depressing activity is additive, since in cultures growing in the presence of them all, no spores could be detected in 10^8 cells (102, 180).

The facts are thus consistent with the following working hypothesis. Sporulation is repressed by nitrogen-containing metabolites, just as the synthesis of histidase is in *Salmonella* (112); the level of this repression would then reflect the intracellular concentration of these repressing metabolites, of which not all carbon and nitrogen sources are equally fast suppliers. The intracellular concentration of repressors may vary in individual cells, particularly when grown in a not very rich medium. Therefore, not all cells will have the critical concentration.

It could be argued that the nature of the nutrilites cannot be varied without also changing the growth rate, and that repression really is a function of that rate. That it is not so has been shown by varying, within physiological limits, the temperature at which cultures growing in various media are incubated. In a given medium, a *B. subtilis* cell is equally likely to sporulate at high and low temperature (and growth rate). In spite of the higher growth rate, it is more likely to sporulate in minimal medium at the higher temperature than in amino acid-containing medium at the lower temperature (180). Experimental tests further supporting the proposed interpretation will be encountered as we go along.

First thought of as a nonspecific "glucose inhibition," catabolic repression (112) now appears to reflect the activity of a regulatory system on the synthesis of catabolic enzymes, functioning in much the same way as the operon-specific end-product repression of anabolic enzymes (105; *see* 45, however). In operons regulated by both catabolic repression and an operon-specific system, like the *lac* operon of *Escherichia coli*, the two systems act in an entirely independent manner (104, 115, 116). Catabolic repression has its own regulatory gene, *CR*, which mutates independently from the operon-specific *lacI* gene, and is believed to produce a cytoplasmic repressor, activated by a catabolite (105). No messenger ribonucleic acid (RNA) is made from a catabolically repressed operon (134). This blocked transcription might only be a secondary effect of a translation blocked directly by the catabolite (*see* reference 45).

The lack of specificity of catabolite repression has been challenged; in the repression of distinct enzymes, the participation of distinct catabolites has been demonstrated (112). A catabolite represses only the enzymes directly or indirectly producing it (117). This new picture of catabolite repression will help us interpret the nature of some of the mutants described later.

Biochemical Aspect

The reader is referred to the reviews cited in the Introduction for information on the antigenic and intracellular enzymatic changes taking place during sporulation. Special attention will be paid here to the extracellular products of the sporulating bacteria. Proteases and nucleases being among the excreted products, turnover of RNA and proteins will be considered first.

Turnover of RNA. At t_0, net RNA synthesis stops abruptly, and total RNA per cell slowly decreases afterwards (61, 194, 203, 240, 241). Some synthesis persists, however (14, 65, 194), in both nuclei (166); it depends on amino acids (15), acting as unspecific carbon sources (65), and it barely compensates for degradation. Even the RNA species which are stable during growth participate in this turnover (14, 16, 194). New kinds of mRNA (messenger RNA) molecules, not produced during growth, appear early in sporulation (4, 56).

Among the mRNA molecules produced at the onset of sporulation, some, detected by Aronson (4) as being relatively stable, might play an important role in the commitment phenomenon; this stability could not be detected in *B. subtilis* however (14, 194). It has been demonstrated, with differentiating slime molds, that commitment does not necessarily mean the end of transcription (106).

Turnover of proteins. "Under conditions of starvation, when no net synthesis is possible, turnover (of protein and RNA) is probably the only mechanism available for achieving adaptation." This remark (114) applies in the case of sporulation, during which protein turnover has indeed been observed (7, 64, 129), and is reflected in the

changes occuring in the antigen makeup of the cell (13).

Not all protein species are degraded at the same rate during sporulation (64). The same is true of *E. coli* during starvation (150, 151, 222); some proteolysis was found to occur even during growth, and Pine proposed that additional classes of proteins are submitted to degradation by "the proteolytic system" of the cell when the medium becomes exhausted. Equally plausible would be the hypothesis that under starvation a derepressed synthesis occurs of new kinds of proteases, which attack new classes of preexisting proteins. The multiplicity of proteases synthesized by sporeformers is now well documented and will be discussed later. The various deprivations (of phosphorus, carbon, or nitrogen) which in sporeformers trigger sporulation (70), also increase protein degradation in *E. coli* (222).

Where in the sporangium are the new proteins synthesized? The location may be in the mother cell or in the prespore, depending on the enzyme being considered (7); this is in keeping with the fact that RNA synthesis in the sporangium occurs at similar rates in association with both nuclear bodies (166). Enzymes nonessential to spore formation can still be produced during sporulation (7); suggestive evidence that some spore proteins, the coats, are synthesized in the mother cell (18, 147) is discussed in the succeeding pages.

Intracellular metabolites. Besides dipicolinic acid and *N*-succinyl glutamic acid, a few new low-molecular-weight compounds, found only in sporulating cells, have recently been described. The activity of one of them, sporogen (chemically unidentified), seems particularly interesting; in a medium not supporting growth, it promotes sporulation in nongranular cells which in its absence would lyse (195, 196).

Also to be mentioned here is the case of some branched (ante-iso) fatty acids, which are present only in sporulating cells or spores (41). This draws attention to important changes occuring in the composition of phospholipids. In bacteria, these compounds are exclusively located in the cytoplasmic membrane, the role of which is conspicuous in spore formation (62).

Extracellular products. The multiplicity of the biologically active substances excreted by sporeformers into their culture medium is well known, but remains unexplained. In bacilli, which will be considered first, these are mainly antibiotics and exoenzymes; in clostridia, they include toxins.

Antibiotics. It was already known in 1949 that bacilli produce many antibiotics (1). Too nephrotoxic to be widely used in therapeutics, their physiological study was first neglected, but their chemical study revealed many striking facts. Many of them are cyclic peptides, containing unusual amino acids, often with the D configuration; some peptidic antibiotics behave like epoxides (160). As a consequence of their odd composition, the peptidic antibiotics are resistant to proteases—at least to those of animal origin usually tested. Like the cell wall peptides, but unlike cytoplasmic proteins, they are not synthesized on ribosomes from aminoacyl-transfer RNA (33, 66).

At least some of these antibiotics are species-specific, and one species may produce several. *B. subtilis* is a good example: in addition to antibacterial antibiotics (1), this species also produces several antifungal ones (113, 185). Among the antibiotics described, it is impossible to know from the literature just how many are produced by one and the same strain, but one strain was shown to produce at least three (139).

No attempt will be made here to review the problem of the mode of action of these antibiotics on sensitive bacteria, beyond saying that many cell functions are affected by one antibiotic or another: cell wall synthesis, oxidative phosphorylations, membrane permeability, etc. More to the point is the question of the physiological meaning of antibiotic production in the life cycle of the producer organism. An important remark in this respect is that antibiotics are never produced by rapidly growing bacilli (25, 26, 227, 229). In the case of bacitracin production by *B. licheniformis* [studied by Bernlohr and Novelli (27–29)], the authors concluded that the antibiotic molecule is a subunit of the spore-coat protein and is incorporated as such in the late stages of spore formation. Mass incorporation of ornithine, a component of bacitracin, into spore coats was not confirmed by Snoke (189), however. Antibiotic incorporation into spore coats has been claimed in the case of circulin (G. J. Jann and H. H. Eichorn, Bacteriol Proc., p. 13, 1964), but could not be detected with either polymyxin (39) or mycobacillin (32). Whether or not mass incorporation occurs, the notion has been introduced that antibiotic production may be an essential step in the process of spore formation.

Proteases. Culture filtrates of sporeforming bacteria, usually studied after the end of growth, have strong proteolytic activity (50, 152, 223). A correlation between protease production and spore formation was suggested when kinetic studies carried out with *B. larvae* (84), *B. licheniformis* (23), *B. subtilis* (48, 120), and *B. cereus* (102) showed this activity to increase rapidly at the end of growth as a result of de novo synthesis, rather than as a result of delayed excretion or activation of preformed enzyme (23, 48, 102, 119). A single enzyme was first assumed responsible for the observed activity and, in con-

trast to intracellular proteolytic enzymes (8, 42), it seemed controlled by amino acid repression (42, 43, 138).

It was later realized that several proteases may be excreted by sporeforming bacteria. This was an already familiar notion with clostridia, because collagenase and elastase activity is easily detected in the presence of other proteases. With bacilli, endopeptidases of the two types described by Hagihara (74) and Morihara (130) may be produced: a metal-enzyme active at neutral *p*H and an alkaline serine-enzyme endowed with esterase activity. This is true at least of *B. subtilis* (73, 111, 121, 153; J. Millet, *unpublished data*) and of *B. licheniformis* (75). Only the neutral enzyme seems to be excreted by *B. megaterium* (126, 127) and *B. cereus* (102), however.

Since in complex media the overall protease activity first appears after t_0, one is led to suspect that with *B. subtilis* the synthesis of both enzymes is controlled by the same mechanism. This was recently tested by Millet, who found the ratio of the two activities to be nearly constant between t_0 and t_4, when the total activity sharply increases (*unpublished data*). Amylase, ribonuclease, and overall protease activities had already been shown to increase parallel with each other (48).

RNA-degrading enzymes. Two RNA-hydrolyzing enzymes have been isolated and characterized in culture filtrates of *B. subtilis*: a neutral ribonuclease, appearing at the end of growth (48, 117, 144), and a phosphodiesterase, degrading also deoxyribonucleic acid (DNA), both native and denatured (93, 135, 148). The specificity of the latter enzyme in vitro has several interesting features which might make it a versatile tool in the living cell.

The isolation of guanosine-5′-diphosphate in culture filtrates suggests that polynucleotide phosphorylase also may be excreted (52). At least the neutral ribonuclease is inhibited by a protein found in cell extracts (188).

Mutants deficient in extracellular ribonuclease activity have been described in *B. subtilis* (97); the residual activity that they still possess seems easily explained by the plurality of the enzymes attacking RNA. More will be said about such strains when sporulation mutants are described.

Wall-lytic enzymes. The presence of an autolysin in the medium of sporulated cultures, as opposed to growing cultures, of bacilli has long been known (68). The autolysin of *B. subtilis* is a true exoenzyme (145), produced shortly after the end of growth, before lysis of the sporangia sets in. Norris detected its production *during* growth in *B. cereus* (146), but this was on solid medium where the physiological age of the population is heterogenous. These early observations are difficult to interpret today. Several lytic enzymes have now been shown to be associated with the cell wall of sporulating bacteria and to be liberated by their autolysis (199; S. L. Kingan and J. C. Ensign, Bacteriol. Proc., p. 30, 1967). An *N*-acetylmuramyl-L-alanine amidase is among them (236). The only lytic enzyme shown in *B. subtilis* to be truly extracellular is lysozyme, an α-*N*-acetylglucosaminidase (156, 157); it is produced during growth, but at a constantly increasing differential rate. This seems to be precisely the kinetics observed with three other exoenzymes (48). Similar data with the other lytic enzymes are lacking. Whether lysis of the sporangia is carried out by the same enzyme(s) as that induced in vegetative cells by starvation (128) or by an anaerobic shock (145) is also unknown.

Amylase. A single exoenzyme, α-amylase, is responsible for the amylolytic activity of the culture filtrates of *B. subtilis* (152). Once disputed, the time course of its appearance is the same as that of the other exoenzymes (48, 96, 145, 226).

Lethal toxins. A variety of substances injuring animal cells (hemolysins, phospholipases) or tissues (collagenase, elastase) are excreted by sporeforming bacteria. Only those with an established lethal activity on whole animals will be considered, this arbitrary limitation being justified partly by the scarcity of information and partly by the incompetence of the writer as a pathologist. Why do so many clostridia elaborate exotoxins, and what is the role of these proteins in the physiology of the cells producing them? These questions remain unanswered (216). The time at which toxins first appear, in cell extracts and in culture filtrates, is an important preliminary question to which there is no uniform answer. Tetanospasmin does not appear until the phase of active growth is over (223). According to Raynaud et al. (154), the same is true of the toxins produced by *Clostridium botulinum*, *C. histolyticum*, *C. sordellii*, and *C. hemolyticum*, but not of that of *C. oedematiens*; also, the time elapsed between synthesis and excretion may not be negligible (*C. perfringens*). The picture is further complicated, in some cases, by the possibility that an increase in toxicity is due not to de novo toxin synthesis, but to activation of a toxin precursor by proteases (35, 100, 101). Toxic activity in filtrates is sometimes said to increase when the cells autolyse (34, 36). It is not clear whether the autolysis referred to is sporangial lysis after sporulation. In conclusion, toxin production in clostridia may occur, in some species, only after the end of exponential growth,

but information obtained from kinetic studies alone is insufficient to settle the point.

Nishida and his collaborators compared toxin production in many strains of the same *Clostridium* species, isolated from heat-treated soil samples. They concluded that the more severe the heat treatment, the lower the production of toxin by strains isolated from the sample. This conclusion applies to *C. perfringens* (233), *C. novyi* (141, 142), *C. sordellii* (143, 211), and *C. tetani* (170), but apparently not to *C. histolyticum* (140).

Stimulating as it is, this conclusion raises many questions, the most obvious being: what is the heat treatment doing? If it is assumed that all strains present in soil had sporulated equally well, then heat must have selected the strains forming the most heat-resistant spores (or forming spores activated by heat), and the relationship between heat resistance of spores and toxin production is an inverse one. This seems to be the case at least with *C. perfringens* (137, 219). But it may also be assumed that oligosporogenous strains are present in soil, along with sporogenous ones; being a small minority, their spores, although heat-resistant, might escape detection after heat treatment. Selection by the latter would then favor strains with a high frequency of sporulation, and again we would have an inverse relation, but now between toxin production and ability to form spores. This seems to be the case with *C. novyi* (142). The case of *C. histolyticum*, in which *a direct* relationship between toxin production and ability to form spores is observed (140, 184), remains a different one. Confronted with an apparently complex situation, Nishida et al. made no comment on the possible nature of the various correlations observed.

A word may be added on toxin production in bacilli. In *B. cereus* (92), a soluble, lethal toxin is produced at the end of growth, at least when *p*H changes, announcing a pending sporulation, occur. (Sporulation itself was not studied.) In *B. cereus* var. *alesti* and in *B. thuringiensis*, an intracellular protein crystal forms, which requires partial digestion in the gut of the insect host to reveal its lethal property. Crystal formation only occurs during the early stages of sporulation (129, 241). The case of *B. anthracis* will not be considered; the recent finding that as many as three distinct factors cooperate in the toxicity of culture filtrates (19, 197) complicates the picture so much that it cannot be profitably discussed in the present context.

Miscellaneous. A nucleotidase (53), a γ-glutamyl transferase (Fukumoto, *cited in* 226), and a hemolysin (22) have also been noted in culture filtrates of bacilli. RNA (52) and DNA (12, 59) are excreted by *B. subtilis* in the apparent absence of cell lysis; the physiological meaning of these facts is unknown.

GENETICAL ASPECT: SPORULATION MUTANTS IN EUBACTERIA

"All this to illustrate that when you have mutants you are better off than when you don't."

(The foregoing was a remark by S. E. Luria, which he considers too trivial to go into print.) As it well appears from the exhaustive review written by Murrell in 1961 (132), and in spite of one notable exception (214), the impact of biochemical genetics was not felt in sporulation research for a long while. Truly, genetic recombination was not known to occur in sporeforming bacteria, but a good deal might have been learned from a systematic study of asporogenous mutants, discovered in *B. anthracis* as early as 1890 (162). In 1958, when attention was drawn by Spizizen to the ability of the Marburg strain of *B. subtilis* to be transformed (176, 190, 191), interest in these mutants increased and a search for them was started independently in Lundgren's (31, 107, 109) and in my laboratory. The later discovery of transducing phages added a new tool for genetic analysis (85, 206, 207, 213).

Isolation of the Mutants

Were it not for their relatively high frequency, sporulation mutants would be hard to find; methods for selecting them are not a priori obvious.

Random picking of mutants; sp$^-$ vs. osp strains. Melanins accumulate in the spores of the Marburg strain. This facilitates the isolation of mutant colonies, which on nutrient agar remain unpigmented, whereas the wild type turn brown (89, 177). Two types of white colonies are observed, opaque and translucent; sporulation is blocked at an early stage (stage zero) in mutants forming translucent colonies. [Even in species forming nonpigmented spores, asporogenous mutant colonies may be recognized by their greater transparency (10, 18, 184).]

Some of the white-colony mutants never form a heat-resistant spore, except by reversion, and are properly called asporogenous (sp$^-$); others, called oligosporogenous (osp), behave as leaky mutants and sporulate at low frequencies (37, 65, 177). All degrees of leakiness and colony shade are observed. At the extremes, osp strains may be difficult to distinguish from either sp$^+$ or sp$^-$ strains.

Melanin synthesis itself may be lost by mutation, leading to sp$^+$ mel$^-$ albino mutants; it may

also be maintained in some sp⁻ mutants, blocked at one of the latest stages of sporulation (J. Millet, *unpublished data*). Clearly a dispensable step in spore formation (177, 179), melanin formation is nonetheless a useful guide in the isolation of sporulation mutants from *B. subtilis* Marburg.

Selection of mutants, and the catabolic repression hypothesis. If the hypothesis of catabolic repression, as discussed earlier, is sound, adaptation to a new carbon or nitrogen source ought to begin with a reduction of the intracellular concentration of the corepressor(s), which might cause sp⁺ cells to become committed to sporulate. Any noncommittable mutant present in the population should then grow freely after adaptation and be selected out. The outcome of the adaptation experiments is obscured somewhat by the fact that not all the cells ever sporulate in the parental sp⁺ population; however, the expected selection takes place and, in the case of adaptation to nitrate utilization as a nitrogen source, is extremely effective (123, 124). Its occurrence is interpreted as further support of the catabolite repression hypothesis.

It is generally known, as an empirical method, that sporulation mutants accumulate in old cultures of bacilli that are kept aerated after sporulation is completed. When this selection is followed against time, a 100-fold increase in the mutant population occurs after autolysis of the sporangia, which must supply new nutrients for growth of noncommitted cells. Thus, the selective mechanism at work in aging cultures is apparently the same as that in the adaptation experiments (124). In a third variation on the same theme, also resulting in mutant selection, glucose is added back to a sp⁺ culture after commitment (10).

To examine the validity of the catabolic repression hypothesis further, two more tests have been used. In the first test, suggested by the fact that in *E. coli* catabolic repression of β-galactosidase synthesis is relieved by a shift to anaerobic conditions (47, 55), the assumption was made that a short anaerobic shock might derepress sporulation, even in a nonexhausted medium. No derepression was observed, however, when the experiment was tried with *B. subtilis* Marburg, a strain which in nutrient broth is relatively insensitive to anaerobic lysis (*unpublished data*); a facultative anaerobe, like *B. cereus*, might be better suited to the purpose of the experiment, however.

In the second test, the behavior in the chemostat of a sp⁺ ade⁻ (adenine-requiring) strain of *B. megaterium* was investigated. In the test tube, this strain sporulates in the absence of both adenine and glucose, but not when adenine alone is missing. In the chemostat, growth at low rates could easily be maintained for several days when adenine was the rate-limiting factor, but when either glucose or ammonium (the nitrogen source) was limiting, sporulation set in and the chemostat was washed out. No washing out was observed under the latter conditions, however, when an sp⁻ derivative, isolated after selection, was substituted for its sp⁺ parent strain. These are the expected results if sporulation is repressed by nitrogen-containing metabolites (11, 180).

Catabolic repression of sporulation, suggested by these physiological observations, received strong support from enzymological work. In bacilli a functional Krebs cycle is dispensable for growth, but not for sporulation (63, 81); enzymes of this cycle can thus be thought of as sporulation-specific. Repression of their synthesis shows "a dual requirement for a glucose (or glycerol) catabolite and an organic nitrogen source" (79, 204). Similarly, the three enzymes of the arginine degradative pathway, detectable only in sporulating cells, are catabolically repressed during growth; they have not been shown to be sporulation-specific, however (95). The regulatory function of metabolites, in sporeforming organisms, is not restricted to enzyme repression and inhibition. In addition, at least in vivo, they increase the activity of some allosteric "growth enzymes", a phenomenon termed "metabolite control" by Leitzman and Bernlohr (99). Thus, exhaustion of the growth substrate may simultaneously derepress the synthesis of sporulation enzymes and decrease the activity of some growth enzymes.

Induction of mutants and the sporulation-episome hypothesis. Some sp⁻ mutants grow normally and do not revert. The sporulation function is thus dispensable. It has been speculated that the entire genetic information specifically required for sporulation might be carried by an episome, which becomes autonomous and expresses itself under conditions of starvation and returns to the inactive integrated state during spore germination (91). Difficulties were encountered in my laboratory, however, when attempts were made to demonstrate the validity of the hypothesis. The episome had to be noninfectious, and all attempts to cure the sp⁺ strain of its hypothetical episome failed, including acriflavine treatment (*see* discussion in reference 172). Pale brown colonies of osp mutants were often obtained, however, the appearance of which could not be explained. The sporulation-specific genetic information obviously was still present in these osp mutants, even if it was only rarely expressed. Since the hypothesis was not invalidated by these negative results, another approach was resorted to: the chromosomal mapping of various sporulation markers.

Successful curing by acridine orange treatment was later claimed by Rogolsky and Slepecky (161). Under the conditions described, it could not be repeated in my laboratory; killing was high and no mass curing occurred. A thorough reinvestigation by Bott and Davidoff-Abelson (37) may explain these discrepancies. With a purified dye of reduced killing activity, used in sublethal concentration, cultures were obtained containing over 20% of mutant cells. The clones isolated again were exclusively of the osp type, but some of them had very low frequencies of sporulation. Up to seven distinct classes could be distinguished among them. The impaired respiratory activity of the mutants obtained, the necessity for their appearance of several divisions in the presence of the dye, and the poor reproducibility of the curing experiment are emphasized in the paper. Bott's suspicion is that the dye exerts its action by preventing the multiplication of a membrane-bound respiratory component (37). Acridine yellow has recently been shown by Stewart to induce gene mutations in *B. subtilis* (198). The frequency of induced mutations observed by Stewart is much lower than the frequency of appearance of acridine-induced osp strains, however. Acridine dyes may thus still have, on some sporulation genes, an additional and specific effect.

J. Northrop and R. A. Slepecky exposed spores to dry heat, under conditions leading to low killing (Bacteriol. Proc., p. 16, 1966), as another means of curing sp^+ cells of a cytoplasmic element. Few auxotrophs, but many (10%) white-colony mutants, were found among the survivors; the mutants were not characterized further. These results were easily confirmed in my laboratory, but, again, most of the mutants were osp strains. Thus, in spite of many efforts, no conclusive evidence has been produced concerning the validity of the initial hypothesis, but the mode of action of the "curing" treatments has raised new and unsolved problems.

Indirect isolation of mutants. Mutants of various kinds have been found to be affected also in their ability to form spores; among those are an aconitase mutant (78, 204), a mutant requiring organic sulfur for growth (108), the mutants resistant to high concentrations of actinomycin D (187), the cytochrome *a*-deficient mutants (H. Taber and F. Sherman, Bacteriol. Proc., p. 31, 1966) and the hemin auxotrophs (3). The functions altered in all these strains would not seem to be sporulation-specific. Other cases of indirect selection will be discussed.

Cytological Classification of the Mutants

Examination of the sporulation mutants in the electron microscope at t_8 or later makes it possible to classify them cytologically (172). This is also true of the osp mutants, since in most osp populations the sporulating cells are too few to be detected by direct examination. In the description to follow, the *selected mutants* will be opposed to the *unselected*; it may thus be useful to recall that both are isolated as white colonies on nutrient agar, the latter from cultures growing in broth and the former from cultures submitted to one of the enrichment procedures described in a previous section. Results with *unselected* mutants will be considered first.

Classification was found to be possible (167) and applicable even to osp mutants (since in most osp populations the sporulating cells are too few to be detected by direct examination). Mutational blocks at all stages of the normal process can be found in *B. subtilis* (167, 169, 179), *B. cereus* (58, 61), and *C. histolyticum* (18) when a large enough collection of mutants is examined. The classification so obtained greatly facilitates subsequent biochemical and genetical studies of the mutant strains (61, 169, 175, 179). The stage at which sporulation is blocked (Fig. 1A) is conveniently indicated as a Roman number in subscript to the spore character of the strain (e.g., sp_{II}^-, osp_{III}, etc; 169).

In our collection of white-colony mutants of the Marburg strain, blocks at stages I, IV, and V are few. In *C. histolyticum*, however, stage V mutants are frequently encountered among transparent colonies (18, 182). The relative frequency of mutants blocked at a given stage might be a reflection either of a bias introduced by relying on colonial aspects for the isolation of mutants, or of the number of enzymatic steps required to reach the next stage. Recently, J. Millet (*unpublished data*) found that some "late" sp^- mutants of the Marburg strain are producers of melanins. Thus, the picking of white colony mutants introduced a bias in the isolation of "late" mutants in this strain.

The situation seems to be different with sp_I^- mutants which, although repeatedly encountered (61, 110, 167), are rare in *B. subtilis*, and could not be found at all in *C. histolyticum* (18). Because the cytological aspect of cells at stage I of sporulation is also seen in growing cells, and because the nuclear rod in some stage I cells has only one mesosome, the justification for maintaining stage I as a sporulation-specific stage due to the fusion of two nuclei has been questioned (168, 169). The recently recognized fact that most cells at t_0 are uninucleated, and thus must go through one last nuclear division (in the absence of cell division) in order to reach stage II, rules out a nuclear fusion occurring at stage I (12).

Among the unselected mutants, there are other

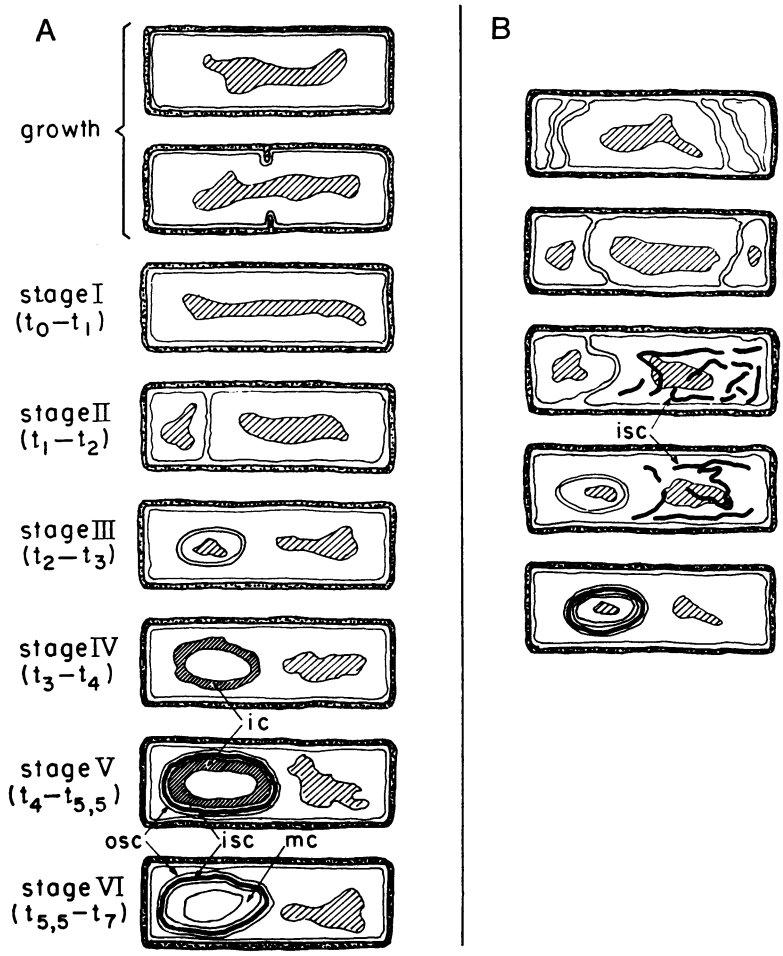

FIG. 1. *Schematic representation of the ultramicroscopic cell structure observed on thin sections of B. subtilis cells at different stages of spore formation (A) and some monstrous* sp^- *mutants at* t_8 *to* t_{20} *(B). (B) From top to bottom:* sp^- *mutant showing anarchic septations:* $spII^-{}_B$ *mutant, forming a spore septum at each pole;* $spII^-$ *and* $spIII^-$ *mutants, forming fragments of the inner spore coat in the mother cell; cortexless* sp^- *mutant. Abbreviations:* ic, *immature cortex;* mc, *mature cortex;* osc, *outer spore coat;* isc, *inner spore coat.*

types than the normal ones just described. In the abnormal mutants, the morphogenetic process visibly goes astray, leading to cellular architectures found only exceptionally in a sporulating, wild-type population. Such are the strains presenting multiple disordered septations, which carve unequal, often nonnucleated cytoplasmic chunks out of the cell (155, 167); or the strains of the so-called $SpIIB^-$ type, which make two normally located spore septa, one close to each pole, but are unable to proceed further with any of them (167, 169, 239). Not all these "monsters", depicted in Fig. 1B, are easy to classify; the difficulty stems from the fact that late structures may be formed, whereas earlier ones are not, suggesting branched sequences of reactions. Such are the cortex-minus (coat-plus) mutants (18, 61, 182), or the stage II or stage III mutants that form dense lamellae with the typical appearance of the internal spore coat in the mother cell (18; Fig. 1B). The pictures strongly suggest that this proteinaceous envelope is coded for by a sporulation gene located in the genome of the mother cell. Cooperation of the two nuclei of the sporangium in the making of a spore is thus indicated; it had already been suspected by Ohye and Murrell (147).

When the selected mutants are similarly examined, they are strikingly found blocked at stage zero exclusively (123, 124, 169). Since mutants unable to become committed should be blocked at stage zero, this result might have been anticipated if the selection methods are truly selecting catabolically hyperrepressed mutants.

The agreement between observation and expectation (123, 124) lends further support to the theory of a catabolic repression of sporulation (180). The selected strains, which are similar in cytological appearance, can be subdivided by physiological tests, however.

No cytological typing has yet been published of the induced or indirectly isolated mutants; unpublished work by Ryter shows, however, that blocks at various stages occur among both the acridine orange mutants of Bott and Davidoff-Abelson (37) and the cytochrome *a* mutants of H. Taber and F. Sherman (Bacteriol. Proc., p. 31, 1966).

Biochemical Classification of the Mutants

General absence of cross-feeding with the sp⁻ mutants. When a collection of asporogenous mutants of the Marburg strain became available in my laboratory, the possibility was investigated that some might feed others and help them to form spores. Although several methods were tried in this investigation, no feeding was observed with any of the mutants studied (175, 179). Identification of the biochemical steps of the sporulation process is hampered by this absence of cross-feeding. Some of the blocked steps can be bypassed by supplying the missing product, however, as the recent isolation, in *C. histolyticum* (182) and *B. cereus* (228), of mutants deficient in the synthesis of dipicolinate illustrates. The latter substance might account for the activity detected by S. S. Witkin and J. M. Kornfeld in germination exudates (Bacteriol. Proc., p. 23, 1967).

Respiratory deficiencies in sporulation mutants. In bacilli, some tricarboxylic acid cycle enzymes (80, 81) and $NADH_2$ oxidase (205) first appear, or greatly increase in specific activity, early during sporulation. This work has already been reviewed (77, 133, 201).

Krebs cycle (204) and cytochrome-deficient mutants (H. Taber and F. Sherman, Bacteriol. Proc., p. 31, 1966) isolated as such, were found sp⁻ as well. Inversely, many sp⁻ mutants isolated from white colonies are deficient in Krebs cycle enzymes (63, 65), $NADH_2$ oxidase (37, 205), or glucose dehydrogenase (37) activity. Deficiencies of this kind would account for the absence of cross-feeding reported in the previous section. It will be interesting to confront this biochemical classification of the sp⁻ strains with the cytological one already described.

Ability to produce antibiotic(s) and exoenzymes in sp⁻ mutant strains of B. subtilis: the various sp_0^- phenotypes. To characterize the ability of sporulation mutants to synthesize the post-logarithmic phase products previously listed, the following phenotypic symbols are adopted. The abilities to produce antibiotic (on *Staphylococcus aureus*), extracellular amylase, protease, wall-lytic, and ribonuclease activities are designated ab^+, amy^+, $prot^+$, lyt^+, and rns^+, respectively. Genetic transformability is termed tfm^+. All these abilities are possessed by the Marburg strain.

There have been isolated sp⁻ strains which are completely or incompletely deficient in one or more of these traits. References are as follows: prot: 37, 65, 102, 175, 192, 193; ab: 17, 37, 175, 179 [Tested on *B. subtilis*, the antibiotic activity reported by Spizizen (192) is distinct from the one detected on staphylococcus (G. Balassa, *unpublished data*).]; tfm: 175, 193, 206, 238; lyt: 175, 193; rns: J. P. Aubert and P. Schaeffer, *unpublished data*. Not a single case of amylase deficiency was observed in the sp⁻ strains studied (175). Recognition of these traits in many mutant clones had to rely on bacteriological plate-tests of admittedly questionable sensitivity and specificity. When all these traits, together with the stage at which sporulation is blocked cytologically in each strain, are taken into consideration, the following conclusions can be drawn (175). (i) All the sp⁻ mutants capable of reaching at least stage II have retained intact all the abilities of the wild type. [Strains with an ab^+ $prot^+$ sp⁻ phenotype have repeatedly been described; the sp⁻ strain producing subtilisin (an alkaline protease; 72) and the sp⁻ ab^+ strain of Shaw et al. (186) may belong in this class of mutants blocked at a late stage of sporulation.] (ii) All the mutants affected in the prot character (more than 50) are blocked at stage zero; the reciprocal is not true, however, a few sp_0^- mutants, said to be of type C (sp_{OC}^-) being ab^+ $prot^+$. (iii) All sp⁻ ab^- strains (more than 50) are also affected in their prot character and blocked at stage zero. Depending on whether the prot character is lost (by the plate test), or merely attenuated, the sp_0^- ab^- strains are said to be of type A (sp_{OA}^-) or B (sp_{OB}^-). (iv) The tfm trait is affected regularly in sp_{OA}^- mutants (over 20 strains tested repeatedly), but only exceptionally in all other mutant types, including sp_{OB}^- and sp_{OC}^-. [Occasional tfm^- strains are similarly found among various auxotrophic mutants; this may reflect the fact that loss of transformability may have more than one cause. The claim that sp⁻ mutants cannot be transformed (206, 238) must have been based on the examination of only a few strains.] (v) Several sp_{OA}^- strains are affected in the lyt character [not all of them, however, as was first thought (175)], and some in the rns trait. [This is consistent with Balassa's finding, that mutants with an impaired turnover of RNA are sp_0^- mutants (16).] (vi) As these conclusions already suggest, the abilities to excrete the various late

TABLE 1. *Some mutant strains and their phenotype*

Strain	ab	prot	tfm	amy
sp^+	+	+	+	+
sp^-_{II} to sp^-_V	+	+	+	+
sp^-_0				
Type A	−	−	−	+
Type B	−	±	+	+
Type C	+	+	+	+

products are not independently lost by mutation. This apparent pleiotropy will be discussed, together with the results reported in the next section. Some of the conclusions are presented in Table 1 (adapted from reference 175).

The list of mutant types appearing in Table 1 is not exhaustive; ab^+ $prot^-$ tfm^+ strains have also been observed (65). Osp mutants are more difficult to characterize than sp^- ones, particularly when they sporulate at a relatively high frequency, because of the heteregenous behavior of their populations; their phenotypes are very similar to those of the sp^- strains described, however (37, 65).

Ability to sporulate of antibiotic-deficient and exoenzyme-deficient mutants of B. subtilis. Most of the sp_0^- mutants, as we have just seen, seem to be deficient in the synthesis of several extracellular products. The finding suggests that these syntheses might be essential early steps of the sporulation process. If this is so, it should be possible to isolate sp_0^- mutants indirectly from the wild-type strain by picking colonies shown on plates to be deficient in the synthesis of one of these products. Moreover, if the latter are not formed independently, as suggested in the preceding section, a mutant isolated as deficient for one extracellular product should also be deficient for others. A study conducted in this fashion (175) confirmed these predictions except in the case of the amy^- mutants isolated, which still had all other traits unchanged. But the mutant strains *fully* deficient for the ab, prot, lyt, or rns characters (admittedly few in each case) were all found to be pleiotropic sp_0^- mutants. (This, incidentally, may be why ab^- strains do not respond to the addition of the crude antibiotic.) Whether isolated as a ab^-, an $prot^-$, a lyt^-, a rns^-, or an unpigmented translucent colony, the sporulation mutants obtained have similar, if not identical, phenotypes. The various activites may not all be completely abolished in every mutant, but as a rule they do not have the wild-type level. The method used for the isolation of a mutant bears no apparent relation to the nature of the characters, which happen to be attenuated rather than suppressed. As an example of the interdependance of the characters studied, the case of a strain producing no detected trace of ribonuclease activity may be mentioned. Isolated as a $prot^-$ strain, in is also $ab^\pm$ $lyt^\pm$ tfm^- and osp_0 (sporulating in broth at a frequency of 1×10^{-6}). The only constant associations so far noted are between the tfm^- and the $prot^-$ (as against $prot^\pm$) traits, and between the ab^- and either the $prot^-$ or the $prot^\pm$ traits (one exception in reference 65, however).

Some mutants isolated as not fully deficient for one trait are also instructive; $prot^\pm$, $rns^\pm$, and $lyt^\pm$ strains were found to be osp_0; five $ab^\pm$ strains isolated as such were sp^+ and normal in every other respect, however. A possible explanation for the latter finding is proposed in a subsequent section.

Production of lethal exotoxin in sp^- mutant strains of C. histolyticum and C. perfringens. The synthesis and excretion of a biologically active protein by a sporeforming bacterium may, as we have seen, be restricted to the sporulation period. This, which seems to apply to many clostridial toxins, prompted an investigation of toxin production by sp^- mutants of clostridia (183, 184). *C. histolyticum* was first chosen as the material (184), since it yields sp^- mutants easily and produces substantial amounts of toxin (600 to 640 minimum lethal dose (MLD) per ml at t_4). In a collection of randomly picked, transparent colony mutants, blockages at all stages of sporulation except stage I were obtained. Most of the mutants were still normal producers of the lethal α-toxin, but three of them produced a maximum of 10 to 20 MLD per ml, and were provisionally called tox^-. When the crude α-toxin from the wild-type strain is studied by immunoelectrophoresis, the presence of two toxic components, α_A and α_B', was revealed. Only component B was present, however, in the supernates of the three tox^- strains, which thus deserve the designation sp^- α_A^- α_B^+.

Determination of the stage at which sporulation was blocked in the various sp^- mutants (18) revealed a remarkable fact: the three tox^- strains are sp_0^- strains whereas the sp^- α_A^+ α_B^+ mutants are blocked at, or later than stage II (184).

Genetic recombination is not known to occur in clostridia. Transducing phages were looked for unsuccessfully, but a search for sp^+ revertants yielded, from one of the tox^- strains, a pseudorevertant osp strain sporulating, under the standard conditions, at a frequency of 1 to 5×10^{-2} (as against $< 4 \times 10^{-7}$ in its tox^- parent strain and 2 to 6×10^{-1} in the wild type). The maximal titer of toxin in the supernate of this revertant was 100 to 200 MLD per ml, and immunoelectrophoresis showed the presence again of component α_A.

A different situation was encountered when

work of this kind was repeated with *C. perfringens* ATCC 3624 (183). Although forming opaque colonies, this organism sporulates at a frequency 10^{-2} in the best sporulation media. In another broth, in which no thermoresistant spores were formed, a large proportion of the cells reached stage II. The designation osp_{II} for the wild-type strain, which describes this behavior best, might lead to confusion, however, because osp_O mutants, sporulating at a frequency of about 10^{-5}, were isolated from it. The designation sp^w was thus adopted instead. Other characteristics of ATCC 3624 were as follows. Titers of α-toxin (phospholipase C) of 20 μg/ml at t_0 and 30 to 40 μg/ml at t_4 were obtained in the best toxigenic medium, the latter figure being the maximal titer; this behavior is called α^+. The hemolytic activity of θ toxin and the overall proteolytic activity, estimated from the size of the halos surrounding isolated colonies on suitable solid media, were recorded as θ^+ and $prot^+$, respectively.

Up to 50% of the colonies obtained by plating wild-type cultures after various acridine treatments showed transparent sectors, seldom seen in untreated controls. Of the strains reisolated from these sectors, about 50% were sp^- (usually sp_O^-, but a few sp_{II}^- were noted), 40% were sp^w and 10% were osp_O. Both hypoproducers (no enzyme at t_0 and, at most, 5 to 8 μg/ml at t_4 to t_6) and hyperproducers (maximal titers of 100 to 1,000 μg/ml) of α-toxin were encountered in *each* of these three classes (183). These results will be discussed in a later section.

Genetics of Sporulation (in B. subtilis)

Genetical classification of the mutants. The genetic analysis of the sporulation process began with the observation that, in a series of (randomly picked) independent sporulation mutants, the ability to form spores can be regained by transformation (177, 178). Sporulation being poor in the usual transformation medium (2), and transformation being poor in sporulation media, the adopted procedure was as follows: After transformation in Anagnostopoulos and Spizizen's medium, the DNA-treated bacteria were transferred into an exhausted sporulation broth containing deoxyribonuclease, and the sp^+ transformants were allowed to form spores before the selective heat treatment was applied (87). By using this quantitative method on several samples of the same competent culture, each treated with DNA from a different strain, the same number of sp^+ transformants is obtained with wild-type DNA and DNA from other sp^- mutants. This being true with many sp^- strains used as recipient, it is concluded that the genes determining the sp^- phenotype are many, and generally unlinked by transformation (177, 179).

When pairs of mutants blocked at the *same* stage are used in the cross, however, some pairs show genetic linkage; this has been observed with sp_{II}^-, sp_{III}^- (163), and sp_O^- mutants (122). Not all the mutants blocked at the same stage are linked (by transformation), however, and as many as 3, 4, and 5 unlinked loci have been detected in sp_O^-, sp_{II}^- and sp_{III}^- mutants, respectively (122, 163). This is interpreted to mean that several biochemical steps are required for a cell to pass from one cytological stage to the next. Although these results raise many unanswered questions, some of which are considered below, they show at least (i) that the mutants can be classified also genetically and that mutations occurring at different genetic locations may block the sporulation process at different stages. The grand total of the sites unlinked by transformation, at which a mutation may lead to a (prototrophic) sp^- phenotype, presently is 15, a figure certainly greatly underestimated; the corresponding number of cistrons is unknown, but cannot be smaller.

Chromosomal mapping of the mutants: the episome hypothesis. The situation created by the sporulation-episome hypothesis and its subsequent developments has already been described. To investigate whether a chromosomal location can be demonstrated for several sporulation markers would seem to be a more direct approach to the problem. (The results just reported bring no information concerning the nature of the genophore carrying the markers employed.) In what follows, the four genetic segments of the *B. subtilis* genome that have already been mapped (57) will be assumed to be parts of just one chromosome, although some doubt on this may still persist. In trying to establish the chromosomal location of the spore markers, two different experimental approaches have been followed.

Takahashi (208–210) started with auxotrophic mutants, of which he isolated sp^- derivatives in a second step. Using preparations of phage PBS1 grown on a prototrophic sp^+ streptomycin-resistant host, he then transduced (206) the double mutants to prototrophy or to resistance. With four of the sp^- strains (sm^s, ser^-, trp^-, and phe^-), he observed a high frequency of cotransfer of the sp^+ trait in the transductants. Four cases of linkage between spore and nonspore markers being thus detected, he studied the frequency of the nonspore markers relative to methionine, following the method of Yoshikawa and Sueoka (235). Assuming a (likely) chromosomal location for the nonspore markers, he thus measured their distance from the origin of duplication and found them to vary from 0.23 (sm) to 0.88 (ser).

Ionesco and Schaeffer (88) started with already mapped auxotrophs, sp^+, and transformed them

to prototrophy with 90 to 95% nitrous acid-inactivated wild-type DNA (103); with two of the strains, phe$^-$ and lys$^-$, they obtained sp$^-$ prototrophs, the sp$^-$ mutations of which (created by the nitrous acid treatment) were linked with the mutant sites of the recipient auxotrophs. One sp_{OB}^- and one sp_{II}^- gene were thus located close to phe and lys, respectively (88). Lastly, an abstract of recent work by M. Rogolsky and J. Spizizen mentions that the mutation in an acridine orange-induced sp_{II}^- mutant is linked to his_I (Bacteriol. Proc., p. 22, 1967). Whether all acridine orange-induced mutations occur at the same site is disagreed upon (*compare* K. F. Bott, Bacteriol. Proc., p. 52, 1967, and M. Rogolsky and J. Spizizen, Bacteriol. Proc., p. 22, 1967), but a single site seems hardly compatible with the multiplicity of the phenotypes observed.

To conclude this section, several reactions, unidentified chemically but required at different stages of the sporulation process, are controlled by chromosomal genes that are unlinked by transformation. The stage at which a mutation will interrupt the sporulation process is thus determined, in some cases at least, by the chromosomal location at which this mutation occurs. The unexplained effects of the acridine or heat treatment cannot be invoked against these facts, which clearly rule out the episome hypothesis in its original form [i.e., the possibility that the *entire* information required for sporulation might be carried by an episome (91)]. Revised forms of the hypothesis are still conceivable in which only a few sporulation genes would be episomal; they will become untenable, however, if it is confirmed that only incomplete blocks are observed in the presumably cured strains (37).

Nature of the pleiotropic sp_O^- mutants. At least two types of sp_O^- mutants have been found to be pleiotropic; those with the ab$^-$ prot$^-$ tfm$^-$ phenotype (122–124, 169, 175, 192), now called sp_{OA}^- mutants, and the sp_{OB}^- mutants, defined as ab$^-$ prot$^\pm$ tfm$^+$ (123, 124, 175). Some, but not all, of the sp_{OA}^- mutants were later found to be affected in the rns or lyt trait as well.

A remark on the use being made of the term pleiotropic may be appropriate here. The sp_O^- mutants are not called pleiotropic because of their deficiency in one extracellular product (which might provoke the sp$^-$ character) while being asporogenous at the same time, but because they are deficient in at least two apparently unrelated extracellular activities. Like other early blocked mutants already described (109, 182), they produce no dipicolinic acid (DPA) but they do not respond to it either. Rather than specifically suppressed by the mutation, DPA synthesis in these strains would seem to stay repressed as a result of some regulatory disturbance affecting, inter alia, an early step of sporulation as well.

Many questions are raised by these intriguing pleiotropic mutants, but the answers as yet are few. The first question, i.e., whether they are single step, revertible mutants, has been answered affirmatively (122). The mutants are detected early, with their full complement of characters, during their selection in nitrate medium; at least some of them revert spontaneously to the sp$^+$ condition, and the revertants have recovered all their lost functions. No segregation of their characters is observed in the transformants obtained when a wild-type culture is treated with their DNA. (122). [This last observation, for unknown reasons, contradicts that of Spizizen, (192).]

The other questions raised by the pleiotropic mutants have no answer yet; they will be discussed in the next section.

Discussion of Sporulation in Eubacteria

The evidence collected in this review illustrates the existence of a direct correlation between sporulation and the production of some exoenzymes, antibiotics, and exotoxins. The crucial point is whether the relation is causal or "fortuitous." Two phenomena need not be functionally related just because they have a simultaneous occurrence. Causality is an appealing working hypothesis, since the finality of the production of these biologically active substances is unknown, and some finality is teleonomically needed (51).

Fortuity on the other hand would not be a fruitless notion if it meant a common origin for both phenomena. Relief from metabolic repression (180) might be this common origin. From a rapidly metabolized growth substrate, such as glucose or glycerol, and in the presence of an utilizable nitrogen source, metabolites would be formed that would repress the synthesis of extracellular enzymes and of at least one early sporulation-specific enzyme, possibly related to the Krebs cycle. The repressing metabolites, which need not to be the same for all these enzymes, should not be produced from the substrates oxidized during sporulation, i.e., organic acids (80, 137) and amino acids (24).

Another suggestion was made by Coleman (48), who thinks the reason for exoenzyme synthesis lagging behind growth is a shortage of RNA precursors while ribosomes are being actively synthesized. The reasoning could be applied to sporulation-specific enzymes as well. The weak point in this argument seems to be that many genes unconcerned with ribosome synthesis become expressed during growth. The dissimilarity

in expression of growth genes and sporulation genes, or exoenzyme structural genes, remains unexplained in this theory, unless a differential (catabolic?) repression is reintroduced, making the theory unnecessary. In addition, determinations of the pool of acid-soluble nucleotides during growth and sporulation (98) show a steady, slow rise, rather than the abrupt increase at t_0 required by the theory. Imprecise as it is (or perhaps because of its impreciseness?), the metabolic repression hypothesis still appears to fit the facts best. It may also be considered a priori that no uniform answer is to be expected in this debate: causality and fortuity might both be encountered, depending on the particular product under consideration.

Exoenzymes, antibiotics, and exotoxins: roles in the sporulation process. Let us symbolically call x, y, and z three postlogarithmic phase extracellular products. In discussing whether their synthesis is an integral part of the sporulation process, the following two rules will be applied. (i) A single x^- sp^+ mutant isolated is enough to rule out causality. (ii) A causal relationship implies not only that all x^- mutants be sp^- as well, but also that they still be y^+ z^+. Otherwise, the mutants would be pleiotropic and probably derived by mutation in a regulatory *CR* gene (105). Their asporogeny might then be accounted for by the disturbed regulation, and should not be construed to mean that synthesis of x, y, or z is an essential step in sporulation. Applying these rules, an attempt will now be made to discuss in each case the possible causality of the observed relationship.

Sporulation is unaffected when α-amylase is suppressed by mutation. The enzyme clearly plays no role in sporulation (rule i). Its synthesis is catabolically repressed, as is further illustrated by the diauxic growth curve obtained when starch is added to a complex growth medium (*unpublished data*). Its function should be that of a food supplier scouting for a new substrate when the medium is approaching exhaustion. Its teleonomic significance is clear and the observed correlation must originate in the common type of repression controlling sporulation and enzyme synthesis.

One difficulty in discussing the role possibly played by proteases in sporulation stems from the fact that cell physiologists (who used mutants and paid attention to the state of sporulation or cell lysis in the cultures studied) assumed a single enzyme responsible for the observed activity, whereas biochemists demonstrated the presence of at least two well-characterized enzymes but did so in filtrates of cultures (of wild-type strains exclusively) merely described as being "24 hours old" or "in the stationary phase", with no reference to sporulation or lysis.

Two types of situation have apparently been encountered, of which *B. megaterium* and *B. subtilis* are good examples. In the former type of organism (126, 127), and apparently in *B. cereus* also (102; N. Angelo and A. Aronson, Bacteriol. Proc., p. 29, 1967), only one enzyme, a neutral endoprotease, is excreted. (Organisms of this type are thus prn^+.) Acting on proteins, this enzyme liberates peptides rather than amino acids (60, 125, 126). Its synthesis, unlike that of intracellular proteases (8, 42), is repressed by amino acids, of which one pair at least is required for maximal repression (42, 43, 102, 119, 126, 127, 138). According to Levisohn and Aronson, amino acids repress indirectly by sparing a protease-repressing metabolite which is, directly or indirectly, an intermediate in many biosynthetic pathways (102). Prn^- mutants have been isolated in which protein turnover and sporulation still occur normally (126, 127; N. Angelo and A. Aronson, Bacteriol. Proc., p. 29, 1967). The first rule applies; the neutral protease is not essential. An intracellular enzyme must be responsible for protein turnover.

In the second type of organism, *B. subtilis*, both neutral and alkaline proteases are synthesized in a coordinate fashion (J. Millet, *unpublished data*); the organism is prn^+ pra^+. An apparently one-step mutation leads to sp_{OA}^- mutants (122), phenotypically prn^- pra^- ab^- tfm^-, and sometimes lyt^- or rns^-, as well. Short of a large deletion (231), which should not be revertible [whereas some sp_{OA}^- mutants are (122)], only a regulatory gene could by mutation have such a pleiotropic effect. Nothing useful to this discussion can be learned from such mutants (rule ii). Analogy with *B. megaterium*, however, would suggest that none of the proteases is essential. Mutant strains of *B. subtilis* deficient in the synthesis of just one protease would be useful; perhaps this is what sp_{OB}^- mutants are. *B. licheniformis*, which excretes three proteases, might belong in the same group with *B. subtilis*.

Extracellular RNA-degrading enzymes have been studied only with *B. subtilis*. At least two enzymes endowed with this activity are known to be produced by this organism. Since overall ribonuclease activity is found to first appear when growth is over (48, 144), this must be true of both enzymes. Only overall activity has been studied with rns mutants. Nothing was said of the ability to sporulate of those obtained by Lanyi and Lederberg (97), but a picture in the article shows their growth on plates to have a translucent appearance, familiar with sp_O^- mutants. The decreased ribonuclease activity of these strains

may have been due entirely to one or the other enzyme. Out of seven strains isolated by J. P. Aubert and myself (*unpublished data*) as rns-deficient on RNA plates, nuclease production was merely belated in one (as was spore formation), decreased in four (osp strains blocked at undetermined stages), and absent in two, which had the sp_{OA}^- phenotype. To conclude, no rns^- sp^+ and no nonpleiotropic rns^- sp^- strain has yet been described and progress will be made only when the various RNA-degrading exoenzymes are tested individually.

Several enzymes are now known to disrupt one or the other bond of the peptidoglycan sacculus. In bacilli, some have been found associated with cell wall preparations and liberated by artificial means; others are known to be released normally. When are these enzymes synthesized, and are they excreted during sporulation? Except for lysozyme, this has apparently not been investigated. Neither do we know precisely which of these enzymes is responsible for lysis of sporangia. According to Chaloupka, at least part of the wall material turns over, even in nongrowing bacteria (44). The same questions can be asked with wall turnover in mind, rather than sporangial lysis.

Only the overall wall-lytic activity of culture filtrates has so far been investigated, comparatively in wild-type and mutant strains of *B. subtilis* (175, 193). Some sp_{OA}^- and sp_{OB}^- mutants have been found to be deficient; inversely, five lyt^- mutants, isolated as such, proved to be sp_{OA}^- or sp_{OB}^- strains as well. Of 14 lyt mutants isolated, none was sp^+ (175). This conclusion is similar to that reached with the nucleases.

In spite of extensive screenings, not all sporeformers are known to produce antibiotics. It may seem unlikely, for this reason alone, that sporulation requires their production. A more careful attitude is needed, however, since not all antibiotics are easily detected. Some are formed but never excreted (227); others, for unknown reasons, are not found in liquid cultures.

One approach to the problem that has been used extensively involves looking for a mass incorporation of the antibiotic into the mature spores. The situation arrived at, reported previously, is rather confusing. This may have several reasons. (i) Antibiotics are extremely diverse chemically and in their mode of action; only a few of them might play a role in spore formation. In a strain producing several, sporulation may not require them all. (ii) Antibiotics might be essential in sporulation by exerting their specific action at some critical time without being eventually incorporated into spores. For this reason, a search for sporulation antibiotics, based on antibiotic-deficient mutants, may be more sensitive than one based on incorporation experiments. This approach, followed with *B. subtilis* in my laboratory, suffers from a great handicap: the nature and the very number of the antibiotics produced are still unknown.

In *B. subtilis*, the one ab^- strain isolated as such was a pleiotropic sp_O^- mutant. No conclusion can be drawn from this single case. Five partially deficient ($ab^\pm$) strains were sp^+ and normal in every other respect. The working hypothesis here is that one nonessential antibiotic out of several produced has been lost in these $ab^\pm$ sp^+ strains. Many more ab mutants should be tested and characterized for each of the antibiotics produced. In conclusion, with the possible exception of circulin (G. J. Jann and H. H. Eichorn, Bacteriol. Proc., p. 13, 1964), no single antibiotic has yet been unambiguously shown to be essential to bacterial sporulation.

That toxin production may be required early in spore formation was suggested by the isolation in *C. histolyticum* of sp_O^- tox^- mutants (184). The hypothesis is consistent with the later findings of Nishida and Imaizumi (140). The demonstration that two electrophoretically distinct components, A and B, participate in the neurotoxicity of the crude toxin, and that component A alone is missing in the supernates of the "tox^-" mutants (184), introduces a complication. The toxins of *C. botulinum* type A (49) and *C. perfringens* (22) have also been found to contain two components. If it is assumed that the toxin is an homopolymeric protein, one component might be another form of the same polypeptide chain, and the mutations observed might have affected its ability to polymerize, rather than its synthesis. When larger numbers of sp_O^- tox^- mutants are isolated, some true tox^- strains might be found. Activation, for instance by a protease, of a protoxin already endowed with some toxicity, is another possibility; tox^- strains might turn out to be $prot^-$ mutants in this case. Whatever the mechanism, the suggestion remains that toxin production is required for sporulation in *C. histolyticum*; this suggestion must now be appraised critically.

Three colonial mutants have been found to be sp_O^- and tox^-, as well. No tox mutants have been isolated directly as such, however, and only those could tell us whether sp^+ strains are found among them. The available information is thus inadequate for this discussion. Since reversion affecting both traits has once been observed in a sp_O^- tox^- strain, it may be admitted provisionally that these strains arose by single gene mutation. Whether the latter affected the structural gene of the toxin, rather than a *CR* gene (105) controlling both sporulation and toxin production, remains

undecided. The previously expressed opinion that toxin is required for sporulation in *C. histolyticum* (184) resulted from a misunderstanding of the basic relations now clearly expressed in the two rules. The situation will be clarified only when tox$^-$ mutants are isolated directly as such in greater number, and this in turn requires that pure antitoxin antibodies be available in large amounts.

Turning now to *C. perfringens*, mention must first be made of a paper by Paquette and Fredette describing the induction of tox$^-$ strains by acridine treatment (149). Here again, toxin production was much reduced in the mutant strains rather than abolished. A hypothetical episome, from which the mutants were supposedly cured, was held responsible for α-toxin production. No attention was paid to the ability of the strains to form spores.

The ability of acridine treatments to induce sporulation mutants suggested an alternative interpretation. If one assumes sporulation in *C. perfringens* to require the production of phospholipase C, then the acridine-induced tox$^-$ strains should be sp$^-$ as well. The work of Sebald and Cassier was started to test this possibility. The outcome was that strains with an unaltered capacity to form spores (i.e., sp^w still) and with a reduced toxic activity were isolated. It is not quite clear whether the first rule applies here, for several reasons. (i) The mutants were isolated on the basis of their colonial appearance, which is not regularly associated with either the tox$^-$ or the sp$^-$ trait. An element of uncertainty may be introduced with this indirect method of isolation. (ii) The toxin should have been estimated serologically as well. (iii) sp^w strains sporulate at low frequency (10^{-2}), whereas some toxin is still produced by the tox$^-$ strains. For these reasons, no conclusion can yet be drawn.

Incidentally, α^{++} strains, producing filtrates of higher than normal activity, have also been encountered after acridine treatment. (Both sp^w and sp$^-$ strains are found among them.) This does not support the curing hypothesis of Paquette and Fredette. Some α^{++} strains, which may be sp^w or sp$^-$, are θ^{++} as well, as if a common regulation mechanism were controlling the production of both toxins. θ^- prot$^-$ strains also have been noted. To conclude, an approach has been followed which has not yet led to an understanding of the observed correlations, but which may already have useful practical applications.

No attempt will be made to extend this discussion to other clostridia. The literature concerning *C. botulinum* has been reviewed in contributions 54 and 55 to reference 86. Toxin location on thin sections of protoplasts, determined with ferritin-labeled type A antitoxin, led to the suggestion that "the spore may play a role in toxin production" (J. J. Duda and J. M. Slack, Bacteriol. Proc., p. 41, 1968).

Finally, in *B. cereus* var. *alesti*, crystalless mutants remain sp$^+$ (239). If the crystal-forming protein is not synthesized in these strains, it is clearly not required for the sporulation of this organism.

To summarize this section, the same, or similar, metabolic repression mechanisms seem to control sporulation and the synthesis of the postlogarithmic products. Some of those have been shown to be functionally unrelated to the sporulation process; a functional relation is still possible with the others. The nature and function of these products are so diverse that no generalization is possible; each must be studied individually.

Problem of the regulation mechanisms controlling the triggering and the unfolding of sporulation. At least 15 genes controlling sporulation have been detected in *B. subtilis*, and about half of them have been mapped. It seems reasonable to expect that both structural and regulatory genes will be found among them. Many of the reactions involved in sporulation still await identification, and we have no way of knowing the dominance relationship between the wild and mutant alleles of the regulatory genes, since no stable heterogenotes have yet been made in sporeforming bacteria. A discussion on regulation in this context can hardly go beyond a priori considerations.

Sporulation soon becomes an irreversible process. It should be profitable to distinguish its triggering from its subsequent unfolding, since only the former is catabolically repressed (64, 69, 70). In *E. coli*, catabolic (105) and operon-specific (90) repressions alike depend on a two-gene regulation system, one of which, the operator, responds to a repressor produced by the regulator gene (*see* 158 and 215, however). [In the summer of 1968, when this review was ready for publication, the two-gene regulatory system proposed by Loomis and Magasanik to account for catabolic repression (105) stood as the simplest model into which the data gathered in this review had to be tentatively fitted. The validity of this model has recently been challenged by Rickenberg et al., however. Their data strongly suggest that the *CR* gene codes for an enzyme of glucose metabolism, rather than for a catabolic aporepressor (215). Thus, ways of thinking have been opened which must be explored and tested before another interpretation of sporulation-controlling mechanism is proposed.] Both derepressed and hyper-repressed (224) mutants are found in operon-specific systems. One may wonder whether mutants with a derepressed or

an hyper-repressed sporulation have similarly been found.

Catabolically derepressed mutants, massively sporulating in the presence of both a growth substrate and a usable nitrogen source, would be lethal unless conditional. Temperature-dependent strains have been looked for in my laboratory, but so far none has been found. Thermosensitive sporulation mutants have been described, but at the permissive temperature sporulation still occurred only in an exhausted medium (107). A mutant strain, isolated after repeated exposures to ultraviolet irradiation, has been observed to produce spores during exponential growth in nutrient broth (202), but the spore-to-cell ratio in the growing culture at equilibrium was about 10^{-2} to 10^{-3}; thus, the strain is far from escaping catabolite repression. Mutants sporulating in high amino acid concentrations, inhibitory to sporulation of the wild-type strain, have been isolated (102), but they grow normally as long as glucose is present and thus cannot be considered catabolically derepressed in the sense given to this expression. In the amino acid-rich medium, in contrast to wild type, the mutants also synthesize protease in large amounts. According to Levisohn and Aronson, amino acids spare a postulated protease-repressing metabolite (PR), derived from glucose but not produced by their own degradation. Protease production and sporulation would be derepressed in the mutants, but only when glucose degradation no longer supplies PR in large amounts. Another interesting question raised by the authors is that of the identity of PR and SR, the postulated sporulation-repressing metabolite. The arguments presented for their being distinct substances seem questionable to the reviewer, who would rather leave the question open.

Let us now examine whether mutants with a hyper-repressed sporulation have been found. In light of recent findings by Fortnagel and Freese (63), it now appears that prerequisites for sporulation (and for postlogarithmic-phase synthesis of exoenzymes) necessarily include (i) building blocks for RNA and protein synthesis. Since they need not be supplied, they must be liberated by enzymes degrading preexisting biopolymers. (ii) They also include ATP, supplying energy for the synthesis of new polymeric molecules. In bacilli, only a functional Krebs cycle, with an intact respiratory chain, will provide enough energy for sporulation (63). (iii) Finally, they include expression of sporulation-specific genes, unexpressed during growth and encoding new species of proteins. Mutations affecting any one of these prerequisites should lead to enzymeless, sporeless, pleiotropic mutants.

Krebs cycle (63, 204) and respiratory (H. Taber and F. Sherman, Bacteriol. Proc., p. 31, 1966) mutants have both been found sp^- as well. Those among them which are affected in the ab or in the prot character are likely to be blocked at stage zero, although this has not been checked cytologically. Data on their pleiotropic nature are scant at best.

Strains isolated as $prot^-$, lyt^-, rns^- could not be distinguished phenotypically from the pleiotropic sp_{AO}^- mutants selected by growth on nitrate. A block in glucose oxidation seems unlikely with these mutants for the following reasons. They grow well on glucose mineral medium, and thus synthesize glutamate from glucose; they also grow (slowly, but no slower than their sp^+ parent strain) on glutamate mineral medium, and thus do not seem to be blocked between α-ketoglutarate and oxaloacetate either (*unpublished data*). Thus, by these criteria also, sporulation and the synthesis of several exoenzymes seem hyper-repressed in sp_{AO}^- mutants, as selection of the latter in the nitrate medium had already suggested. The case of the sp_{OB}^- strains is not as clear yet.

Very little can be guessed of the regulation of sporulation-specific reactions once the process is triggered. An ingenious method for ordering these biochemical events sequentially has recently been devised (G. Balassa, Bacteriol. Proc., p. 25, 1968), and should be useful when clean biochemical tests are substituted for the bacteriological plate-tests. The polypeptide chain(s), of which spore coats are made, are synthesized early during sporulation, but they only appear as morphologically distinct structures on electron micrographs at stage V, when exogenous cystine is incorporated (5). The coat structure still appears in some coretexless mutants (18, 61) and even in mutants blocked as early as stage II (18; Fig. 1B). Some late reactions, like cystine incorporation, can thus still occur when earlier ones fail. This is not always the case, as was previously noted à propos dipicolinate synthesis.

SPORULATION AND PRODUCTION OF ANTIBIOTICS, EXOENZYMES, AND EXOTOXINS IN ACTINOMYCETES AND THE LOWER FUNGI

Endospore formation in eubacteria, conidiation in actinomycetes and in lower fungi, meiosis in yeast, etc., are often designated under the common name of sporulation. Much more than the name is common to these phenomena in spite of the conspicuous differences existing among them; they

all are intracellular differentiation processes, subdividing the cell by neomembrane formation, and remaining unexpressed as long as rapid growth is possible. The basically similar physiological situation is reflected in the striking similarity of the usual sporulation media, empirically and independently worked out for yeast and bacilli. With species of *Penicillium*, the recommended procedure to obtain good sporulation in submerged cultures is to transfer the organism into a medium containing sugars in high concentration, but no nitrogen source; the absence of sugar is conducive to autolysis of the mycelium (131). These considerations prompted a search in the literature, reported in this section, to determine whether the production of antibiotics, exoenzymes, and exotoxins was also correlated with sporulation in actinomycetes and the lower fungi.

Together with the ability to form aerial hyphae (conidia?), colonial morphology variants of *Actinomyces griseus* were found, as early as 1945, to have lost the capacity to produce streptomycin (sm^-); both properties were regained together in the revertant strains isolated (181). Essentially the same situation was encountered when streptothrycin production by *A. lavendulae* was studied (218). These observations should perhaps not be ascribed to the ability of the strains to conidiate, however, but rather to a change in the branching habits of the growing mycelium, since the sm^- mutants described by Szabo et al. (200), although pleiotropic, were sp^+ still.

The incompatibility of cell growth and antibiotic biosynthesis, "a commonplace of fermentation practice" (40), is generally agreed upon (83, 217, 229). The function of "secondary metabolites" (antibiotics and their precursors) may well be "the formation of specialized structures permitting survival under adverse conditions, i.e., the formation of spores, conidia, sclerotia, etc" (40). To quote Woodruff (229), antibiotic production might be "a fundamental property of the differentiating cell," and its raison d'être "the survival advantage conferred to the organism by its differentiation, under conditions adverse to growth." The general impression so expressed, short of a rigorous demonstration, is certainly in keeping with the situation reported with bacilli. The view sometimes expressed that antibiotics might result from inborn errors of metabolism is either ignored or denounced as heretical.

Worth mentioning in the present context, although not endowed with antibiotic activity, are the peptolides (sporidesmin and sporidesmolides) produced at sporulation by certain fungi (54, 164); they are located at the surface layers of the fungal spores (30).

Little information could be found, beyond the mere mention of their existence (46, 217), on protease and nuclease production by actinomycetes, but the case of extracellular wall-lytic enzymes seems better documented (221). Emphasis was put on the fact that these enzymes are produced exclusively by the aerial mycelium when sporulation sets in (220). *N*-acylmuramyl-L-alanine amidase was first identified in the culture filtrates of a *Streptomyces* (67).

Extracellular wall-lytic enzymes are also produced by fungi, together with proteases and nucleases (50, 74). The correlation between protease excretion and sporulation was probably first noted with a fungal enzyme (94). Three distinct proteolytic enzymes are excreted at sporulation by a species of *Aspergillus* (20, 74). The region of protease formation is at a constant distance from the hyphal tip, and thus bears a constant spatial relationship with the membrane site where the first conidiation septum is formed (234). In a *Penicillium* species the causal relationship has been objected to: complete suppression of protease formation could be obtained with little action on sporulation (212). In Takadiastase, a commercial crude enzyme preparation from an *Aspergillus* species, two distinct (extracellular) ribonculeases have been found (171). Lastly, lower fungi produce many toxins, including the well-known aflatoxin (118, 225); their possible relation to sporulation apparently has not been looked into.

SPORULATION MUTANTS IN GENERAL AND APPLIED MICROBIOLOGY

The complex process of sporulation, as this review illustrates, can be chopped down to more easily analyzed pieces by studying asporogenous mutants in large numbers. This is a classical way to conduct the analysis of a physiological process; the only surprise here is that it was not followed earlier. An additional virtue of this approach is that providence often adds some traits of its own to those expected of the mutants. This is what makes a sensible statement of Luria's joke, cited at the beginning of the second part of this review, and justifies (so it is hoped) the otherwise "antiethical" quotation of a reluctant author. The providential traits, in the present case, are in some mutants the inability to produce antibiotics, exoenzymes, and exotoxins and, in others, the ability to produce specific types of monstrous cell structures (Fig. 1B).

What kinds of work could sporulation mutants be put to, besides the analysis of sporulation itself and of its regulation? The search for better pro-

ducers of antibiotics has already been conducted empirically on such a large scale that the present situation is unlikely to be further improved by the uncertain isolation of mutant strains defective or derepressed in their sporulation; a posteriori recognition that already exploited mutants are of such a nature seems possible if such profitable strains were made available to scholars, a condition unlikely to be soon fulfilled.

Antibiotics required for sporulation, if some are found, might conceivably be excreted only in loosely regulated strains; those tightly regulated would then appear to produce none. A search in cell extracts would be rewarding in this case, particularly if carried out comparatively in sp^+ and sp^- strains. Never excreted, gramicidin S might offer an example of such a situation (227).

The mode of action of an antibiotic, when it is known, is generally established on sensitive bacteria, unrelated to the producer organism, and tested in growth-supporting media. One would like to know whether the same action is exerted in the producer by the endogenous antibiotic, a question seldom raised, and one which could perhaps be answered by comparing sp^+ and sp^- strains under conditions conducive to sporulation.

Extracellular enzymes of microbial origin have long been commercialized and their market seems to be expanding. It may be of some economic interest that overproducers of protease (J. F. Michel, *personal communication*) are found among sporulation mutants. Exotoxins are used for toxoid and antitoxin production; mutants producing increased amounts of toxin have similarly been found among sporulation mutants (183).

If some toxins, the "sporulation toxins," are actually required for sporulation, a single biological activity is suggested by Ockham's principle as responsible for both this requirement and the toxicity for animal cells. Pursuing this reasoning further, a comparative biochemical study of tox^+ and tox^- bacteria, incubated in a sporulation medium, should be a simpler way to identify this biological activity than a study of the intoxicated animal.

Asporogenous mutants derived from transformable strains might also be a good tool for studying the building of the competent state, since transformability is usually normal in sp_{OB}^- mutants and deficient in sp_{OA}^- strains; the loss of transformability in type A mutants cannot be ascribed to their $prot^-$ trait, however, since $prot^-$ tfm^+ strains have been observed (65). Autoradiographs of sporulating bacteria, washed with deoxyribonuclease after exposure to ^3H-labeled DNA, frequently show the radioactivity in close association with the forespore membrane (237); in the medium of Bott and Wilson, competence regularly appears at t_3 (38). These observations might suggest that ability to reach stage III of sporulation is somehow essential for transformation to occur; this cannot be so, however, since mutants blocked at an earlier stage can be normally transformable.

Finally, biochemists often seem worried about the instability of enzymes or ribosomes in extracts of *B. subtilis* cells. Inasmuch as this instability may be due to extracellular enzymes present in the extracts, some sp^- mutants, devoid of these enzymes, might be used advantageously.

In conclusion, sporulation mutants appear as versatile tools which have been neglected too long.

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